Microstimulation of the Lumbosacral Spinal Cord: Mapping

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ABSTRACT

The objectives of this research are to define the anatomical locations of neuronal populations involved in control of genitourinary and motor functions, and to determine the physiological effects of stimulation of these neuronal populations. During this quarter we have made progress on both of these objectives. We conducted experiments to map the locations of spinal neurons involved in genitourinary function using expression of the immediate early gene c-fos. We conducted microstimulation studies to map systematically the isometric torques generated about the knee joint by microstimulation of the lumbar spinal cord. We also designed and constructed a new high compliance voltage regulated-current stimulator to use in microstimulation experiments.

INTRODUCTION

Electrical stimulation of the nervous system is a means to restore function to individuals with neurological disorders. The objective of this project is to investigate the feasibility of neural prosthetics based on microstimulation of the spinal cord with penetrating electrodes. Specifically, we will use chemical and viral retrograde tracers, stimulation mapping, and field potential recordings to determine the locations in the spinal cord of the neuronal populations that control genitourinary and motor functions in the male cat. We will use selective microstimulation with penetrating activated iridium microelectrodes to determine the physiological effects of stimulation of different neural populations. The results of this project will answer fundamental questions about microstimulation of the spinal cord, and lead to development of a new generation of neural prosthetics for individuals with neurological impairments. During the seventh quarter of this contract we made progress on anatomical mapping of the neurons involved in genitourinary control, and on measuring the effects in the motor system of microstimulation of the lumbar spinal cord. Below each of our accomplishments is summarized.

PROGRESS IN THIS QUARTER

I. Anatomical Tracing of Genitourinary Innervation

Expression of c-fos in Genitourinary-related Spinal Neurons

The purpose of theses experiments is to determine the location and rostrocaudal extent of spinal neurons that participate in control of micturition. The proto-oncogene c-fos that encodes the Fos protein can be induced rapidly and transiently in post-synaptic neurons by increased electrical activity. In these studies we used expression of Fos protein to identify cells within the sacral spinal cord that regulate genitourinary function in male cats. In our previous c-fos mapping experiments (see QPR 6) we used electrical stimulation of either the pelvic nerve or pudendal nerve to induce c-fos expression. In the present experiments we used neuronal activity arising during isometric voiding to induce c-Fos expression. The results of these experiments identify neurons that were electrically active during the behavior of voiding, and thus identify spinal sites to target for microstimulation.

METHODS Sexually-intact male cats were anesthetized with xylazine (Rompun, 2.0 mg/kg, SQ) and ketamine HCl (Ketaset, 15 mg/kg, IM), a venous catheter was inserted in the cephalic vein, and anesthesia maintained with α-chlorolose (Sigma, 60 mg/kg IV, supplemented at 10-15 mg/kg). In two cases, Ketamine was replaced by gaseous halothane anesthesia (1-2% in O_2), to determine the influence of using an NMDA-antagonist (ketamine) on the patterns of c-Fos expression. Animals were intubated and respirated, body temperature was maintained with a thermostatically controlled lamp, and heart rate was continuously monitored. The skin around the incision site was anesthetized using Marcaine (0.5%, 1 cc injected ID and SQ) in an effort to reduce surgically

induced Fos expression. A ventral midline incision was made to expose the pre-prostatic urethra between the bladder neck and the pubic symphysis. The bladder was cannulated per-urethrally with a PE-50 catheter, and the catheter was fixed in place with a tie around the pre-prostatic urethra. The incision was closed and the catheter was connected to a pressure transducer in series with a constant flow infusion pump. In some cases the electromyogram (EMG) of the periurethral striated musculature was also recorded. A bipolar electrode consisting of two stainless steel wires (0.0018" diameter), insulated except for 2 mm at their tips, was introduced using a 25 ga. hypodermic needle. The EMG electrodes were inserted through the skin ventral and lateral to the anal sphincter with the needle directed toward the midline.

The pressures generated in the bladder were measured using a solid state pressure transducer connected to the per-urethral bladder catheter (Deltran DPT-100, Utah Medical Products, Midvale, UT). The pressure signals were amplified, low pass filtered (fc=30 Hz), and continuously recorded on a strip chart recorder. EMG signals were amplified, filtered (10 Hz-1 kHz), displayed on a storage oscilloscope, and recorded along with the bladder pressure signals on VCR tape. Signals were later digitized off line at 2 kHz.

Warm saline was infused into the bladder (1 ml/min) until spontaneous periodic bladder contractions occurred and then the infusion was stopped. Isometric voiding was maintained for either 2 h (n=2 with ketamine, n=2 with halothane) or 4 h (n=1) after which time the catheter was removed and the animal was able to void. In a control animal, the catheter was placed but no fluid was infused and the animal was free to void through the catheter.

One to 2 hours after the period of isometric micturition, the animals were perfused via the aorta with saline followed by 4% paraformaldehyde in 0.1M $NaPO_4$ (pH=7.4). The brain and spinal cord were removed, stored in fixative for 2-5 days, and then transferred to 30% sucrose in 0.1M $NaPO_4$ for 2-4 days.

The sacral spinal cord was sectioned transversely at 50μm intervals on a freezing microtome. Floating sections were rinsed and exposed overnight to rabbit anti-Fos (1:10,000, Oncogene). After rinsing, sections were exposed to secondary antibody (biotinylated goat anti-rabbit) for 1 h. Fos-like immunoreactivity was visualized with the ABC method intensified with nickel chloride. Sections were rinsed, mounted on slides, dehydrated in alcohol series, cleared in xylene, and coverslipped. Slides were viewed using bright-field microscopy and locations of cells exhibiting fos-like immunoreactivity described according to the conventions of Rexed [1954].

TABLE 1: Summary of experiments on the patterns of c-Fos expression induced by isometric micturition.

ID	Anesthesia	Stimulus
120	Xylazine/Ketamine/Chlorolose	2.0 h isometric micturition
121	Xylazine//Ketamine/Chlorolose	2.0 h isometric micturition
122	Xylazine//Ketamine/Chlorolose	0.0 h isometric micturition (Control)
123	Xylazine//Ketamine/Chlorolose	4.0 h isometric micturition
124	Xylazine//Halothane/Chlorolose	2.0 h isometric micturition
125*	Xylazine//Halothane/Chlorolose	2.0 h isometric micturition

*- this tissue has not yet been analyzed.

RESULTS Infusion of warm saline into the bladder resulted in quasi-periodic contraction and relaxation of the bladder (fig. 1A). During periods of bladder quiescence, there was a large amount of electrical activity (EMG) in the periurethral striated musculature. However, the regular increases in bladder pressure were accompanied by synchronous reductions in the electrical activity in the periurethral striated musculature (fig. 1B). The degree of EMG reduction varied from contraction to contraction, but the EMG activity was consistently lower during contractions of the bladder than during intervening periods. These patterns are analogous to those which occur during non-isometric micturition in the decerebrate cat [Shimoda et al., 1992, Fedirchuk and Schefchyk,

1993], and thus isometric micturition in chlorolose anesthetized cats appears to be a valid method to induce repetitively the behavior of micturition.

In all animals undergoing isometric micturition, nuclei exhibiting Fos-like immunoreactivity (FLI) were found bilaterally in S1-S3, with the largest numbers of cells (~10-60 cells/section) present in S1 and S2. Controls in which the primary antibody was omitted from the processing sequence resulted in no FLI, indicating that there was not non-specific binding of the secondary antibody. There were no obvious differences in the patterns or intensity of FLI in the 2 and 4 hour animals. Similarly, there were no obvious differences in patterns or intensity of FLI in animals receiving Ketamine and the animal not receiving Ketamine (i.e., animals receiving halothane).

Although large numbers of Fos-postive cells were found in the sacral spinal cord, they were restricted in their laminar distribution (fig. 2). Fos-positive neurons were found in the superficial dorsal horn throughout Lamina I, in the lateral portion of lamina II, dorsal to the central canal in lamina X, and dorsolateral to the central canal in the medial part of laminae V and VI. A horizontal band of cells exhibiting FLI was found in laminae VI and VII extending from dorsal to the central canal to the intermediolateral cell column (fig. 2). Large numbers of Fos positive cells were identified in the dorsolateral portion of the ventral spinal cord in the intermediolateral cell column. This region contains the preganglionic parasympathetic innervation of the bladder and urethra, as well as genitourinary-related interneurons. FLI was rarely observed in ventral horn neurons (0-1 cells/section).

The results of these studies identify post-synaptic spinal neurons involved in control of the genitourinary system. The locations of neurons expressing FLI after isometric micturition was more widespread than after electrical stimulation of either the pelvic nerve or the pudendal nerve, although there were several regions in common to all three stimuli (see QPR #6). These results, obtained in cats, parallel previous studies of fos expression in a rat model [Birder and de Groat, 1992] and previous studies using psuedorabies virus to label spinal neurons synapsing upon the preganglionic innervation of the bladder.

II. Microstimulation of the Lumbar Spinal Cord in Male Cats

The objective of these experiments is to map the torques generated about the knee joint by microstimulation of different regions of the lumbosacral spinal cord. In this series of experiments the evoked torques evoked by microstimulation were mapped, in a given rostrocaudal segment, systematically from the midline to the lateral border of the spinal cord at 250 μm intervals. Penetrations were made in rostrocaudal planes between the caudal border of the L4 segment and the middle of the L7 segment.

METHODS Male cats were anesthetized with xylazine (Rompun, 2.0 mg/kg, SQ) and Ketamine HCl (15 mg/kg, IM), a venous catheter was inserted in the cephalic vein, and anesthesia maintained with α-chlorolose (60 mg/kg IV, supplemented at 10-15 mg/kg). Animals were intubated and respiration was maintained with a respirator. A laminectomy was made to remove the L4-L7 vertebrae. The animal was mounted in a spinal frame with pins at the hips, the head in a headholder, and vertebral clamps at L3 and S1. Body temperature was maintained between 37° and 39° C with a thermostatically controlled heat lamp, warm 5% dextrose saline with 8.4 mg/cc sodium bicarbonate added was administered IV (~20 cc/hr), and heart rate was monitored throughout the experiment. Dexamethasone (2 mg/kg, IV) was administered at the completion of the laminectomy and every 6 hours thereafter to prevent inflammation of the spinal cord.

The torques generated about the knee were measured using a custom-built strain gage instrumented beam (described in QPR 2). Torque signals were amplified, low pass filtered (fc=100 Hz), and continuously recorded on a strip chart recorder. Torques evoked by stimulation were also sampled by computer (fs=200Hz).

We used activated iridium wire electrodes purchased from Huntington Medical Research Institutes, Pasadena, CA (50μm Epoxylite insulated iridium wire with an exposed electrochemically determined surface area of ~225 μm^2 and a 1-3 μm tip). Stimuli were charge

balanced biphasic pulses with an amplitude of 10-150 μA and a pulsewidth of 100 μs applied as 1 s to 10 s continuous trains with a frequency between 2 Hz and 100 Hz. Our standard mapping stimulus was a 1 s 20 Hz burst. Vertical, dorsal-to-ventral penetrations (increment=100-400μm) were made at multiple mediolateral locations (increment=250μm) in the middle of the lumbar segments and at intersegmental boundaries. The torque responses were quantified by averaging the torque during the stimulus interval.

RESULTS The results from one experiment in which three rostrocaudal levels of the lumbar spinal cord were systematically mapped are shown in figure 3. Each panel is a two dimensional map of the average knee torques evoked by microstimulation of the lumbar spinal cord (1 s 20 Hz burst of 100 μs, 50 μA or 100 μA pulses) at three different rostrocaudal levels: the middle of L5 (A), the L5/L6 border (B), and the middle of L6 (C). The evoked extension or flexion torques are plotted as grayscale values. Mediolateral zero corresponds to the midline, dorsoventral zero corresponds to the cord surface at the midline, and the intersections of the orthogonal grid lines indicate the points at which mapping stimuli were applied.

These maps demonstrate that net flexion torque was the dominant response evoked by microstimulation at these levels. Net extension torque was evoked only in the lateral aspect of the ventral horn of mid L6. It is important to emphasize that recordings of the net torque do not exclude the possibility of co-contraction. Flexion torques were evoked by microstimulation in the dorsal aspect of the cord, with the largest torques evoked by stimulation in regions around the dorsal root entry zone. These responses were putatively evoked by stimulation of afferent fibers terminating within the gray matter. Flexion torques were also evoked at some locations in the intermediate region of the cord (2000 μm below the surface at the L5/L6 border and in mid L6). This responses may represent microstimulation of excitatory spinal interneurons, which are known to exist in this region [Jankowska, 1992], or stimulation of afferent terminals. The largest torques were evoked in the ventral horn where there was a clear mediolateral segregation between generation of flexion torques medially and extension torques laterally (fig. 3C).

These results support those reported previously in which we demonstrated a mediolateral segregation of flexion and extension torques (see QPR 6), as has been suggested by anatomical studies [Romanes, 1951]. These data also show the dominance of net flexion torque generated by microstimulation, and that flexion torques can be evoked by direct motoneuron activation (by microstimulation in the ventral horn) as well as by transynaptic activation (by microstimulation in the dorsal and intermediate regions). The two-dimensional maps which we have developed are an effective way to present data from microstimulation experiments. However, they demonstrate a shortfall in our experimental protocol. Namely, our "systematic" mapping was not systematic enough. It is clear that both stimuli (50 μA, 100 μA) were not applied at all cord location, as we would like. Further fig. 3C demonstrated that our penetrations were not deep enough to explore the entire ventral horn, particularly at the other rostrocaudal levels. Thus, in addition to being an effective means to present mapping data, the two dimensional data also suggest ways to modify our experimental protocol.

III. A High-Compliance Voltage Constant Current Source for Microstimulation

The high impedance of the small microelectrodes used for intraspinal microstimulation requires a stimulator with a large compliance voltage to maintain regulated current and to achieve stimulus pulses with a fast rise time. The activated iridium microwires from HMRI which we use currently (see previous QPRs for specifications) have typical impedances of ~70 kΩ, but the thin film DEPTH electrodes from the University of Michigan have typical impedances near 1 MΩ. During this quarter we designed and fabricated a two-channel stimulator for use with high impedance microelectrodes.

METHODS The stimulator has a compliance voltage of 200V, and thus can drive 100 μA into a 2 MΩ electrode. It generates biphasic charge balanced pulses with the amplitude of the primary phase selected from a series of switches to generate 25-150 μA in 25 μA steps or alternatively from

a 10-turn potentiometer to generate an arbitrary current from 0 to 200 μA. The duration and frequency of the primary phase are determined by a computer-generated TTL trigger pulse. The amplitude of the secondary recharge phase is adjustable using an internal potentiometer (R21), and the duration of this phase is set automatically to balance the net charge.

Schematics for the circuit power supply, the output power supply, and the stimulator circuit are provided in figure 4. Each channel is powered by a single 9V lithium battery. The 200V output supply is achieved using a step-up switched-mode dc-dc converter (MAX773). The stimulator circuitry is isolated from the computer ground with an optical isolator (HP 6N137), the output of which is normally high. A TTL trigger pulse makes the output of the optocoupler low. This low signal is inverted with Q4 (2N3904) and this high signal turns on the voltage to current converter (TLC272+2N5551) which enables current to flow in the high-voltage output transistor (p-MOSFET VP2410L). When the trigger signal is not present, Q3 (2N5551) is turned on and the recharge current flows through the load.

RESULTS An example of a 100 μs, 100 μA pulse into a 100 kΩ load is shown in figure 5. The 10-90% rise time is approximately 20 μs. The new stimulator enables us to generate fast, regulated current pulses through the large loads imposed by small microelectrodes and provides performance superior to our previous stimulator which had a compliance voltage of only 45 V. The individual isolated channels will enable us to investigate multielectrode intraspinal microstimulation.

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OBJECTIVES FOR THE NEXT QUARTER

I. Anatomical Tracing of the Genitourinary System

We will complete our series of experiments to identify spinal neurons involved in control of micturition by mapping expression of the c-fos protein. We will conduct 3 experiments with microstimulation of Barrington's nucleus in the pons to evoke micturition over a period of 2 hours. These results will be compared to those obtained by 2 hours of isometric micturition evoked by bladder filling.

II. ENG/EMG Instrumentation

During this quarter we began fabrication of a four-channel version of a gated biopotential for use in microstimulation experiments (see QPR 5). During the next quarter we will complete the amplifier and use it to record electromyograms evoked by spinal microstimulation.

III. Microstimulation of the Lumbosacral Spinal Cord

We will continue experiments to map systematically the motor responses generated by microstimulation of the lumbar spinal cord. Specifically, we will incorporate EMG recordings to assess response latencies and selectivity of stimulation, and we will ensure complete segmental mapping with application of all stimulus amplitudes at each location.
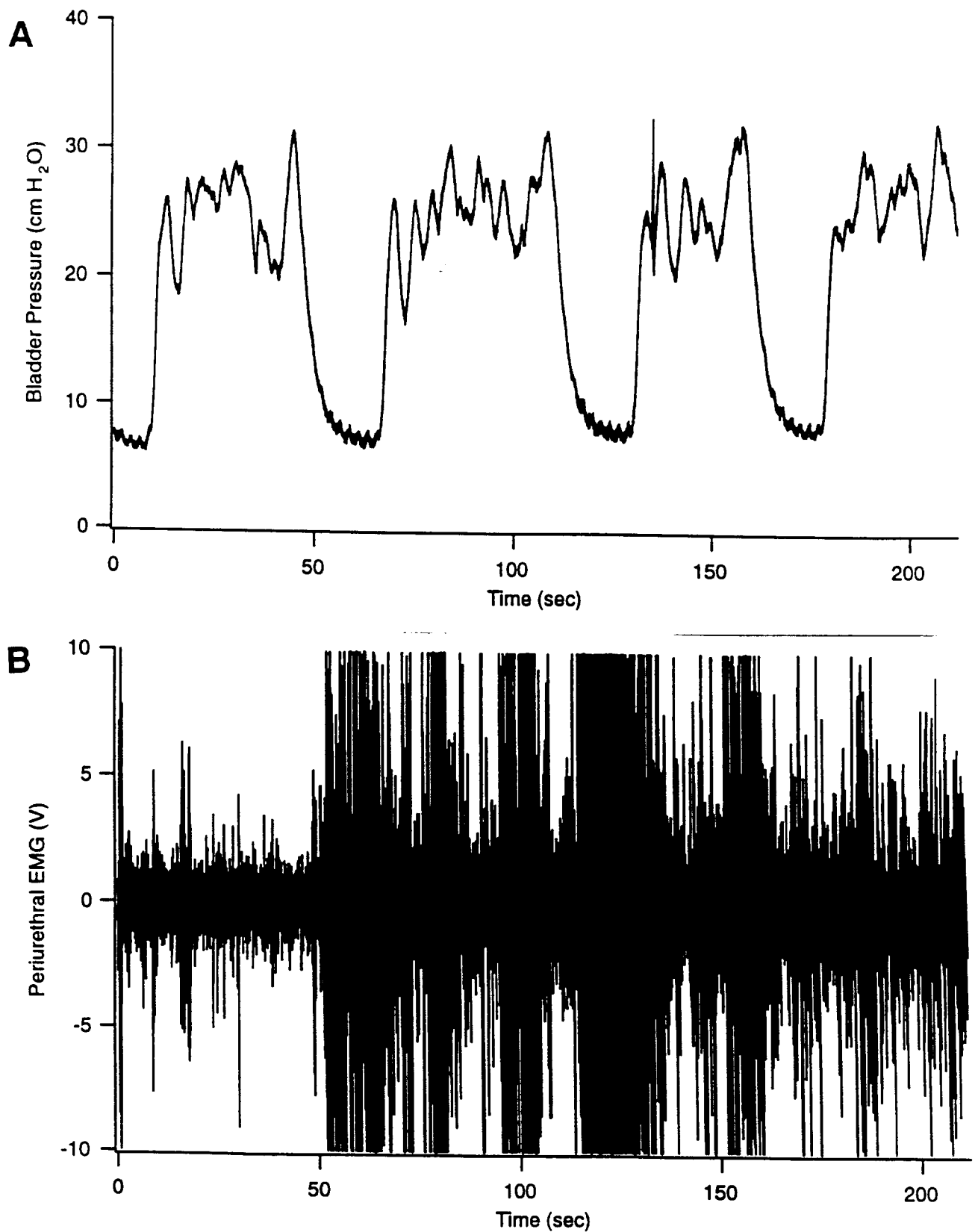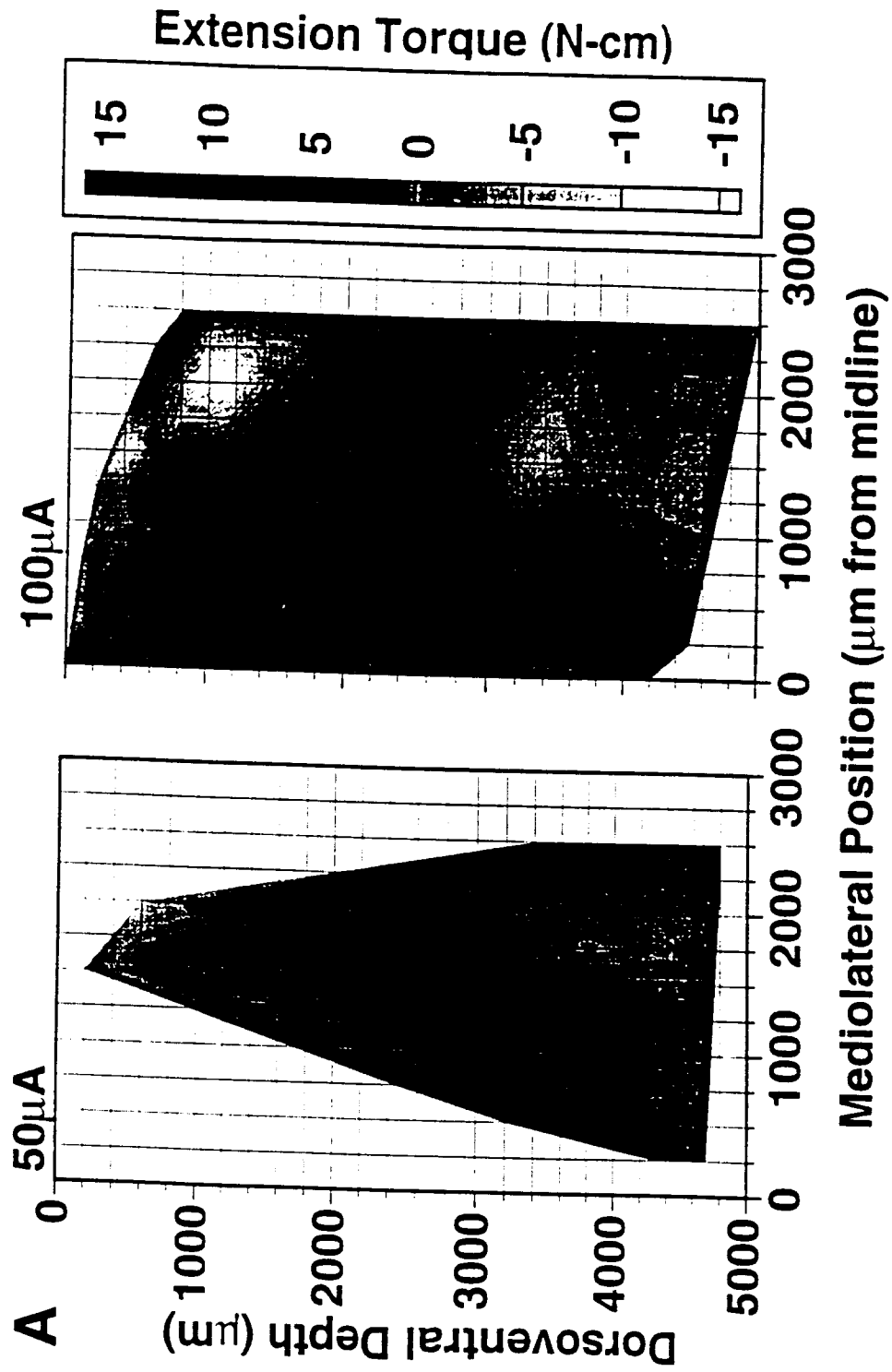
Figure 1: Patterns of bladder pressures (A) and electrical activity (EMG) in the periurethral striated musculature (B) recorded during isometric micturition induced by slowly infusing warm saline into the bladder while the urethra was tied closed.
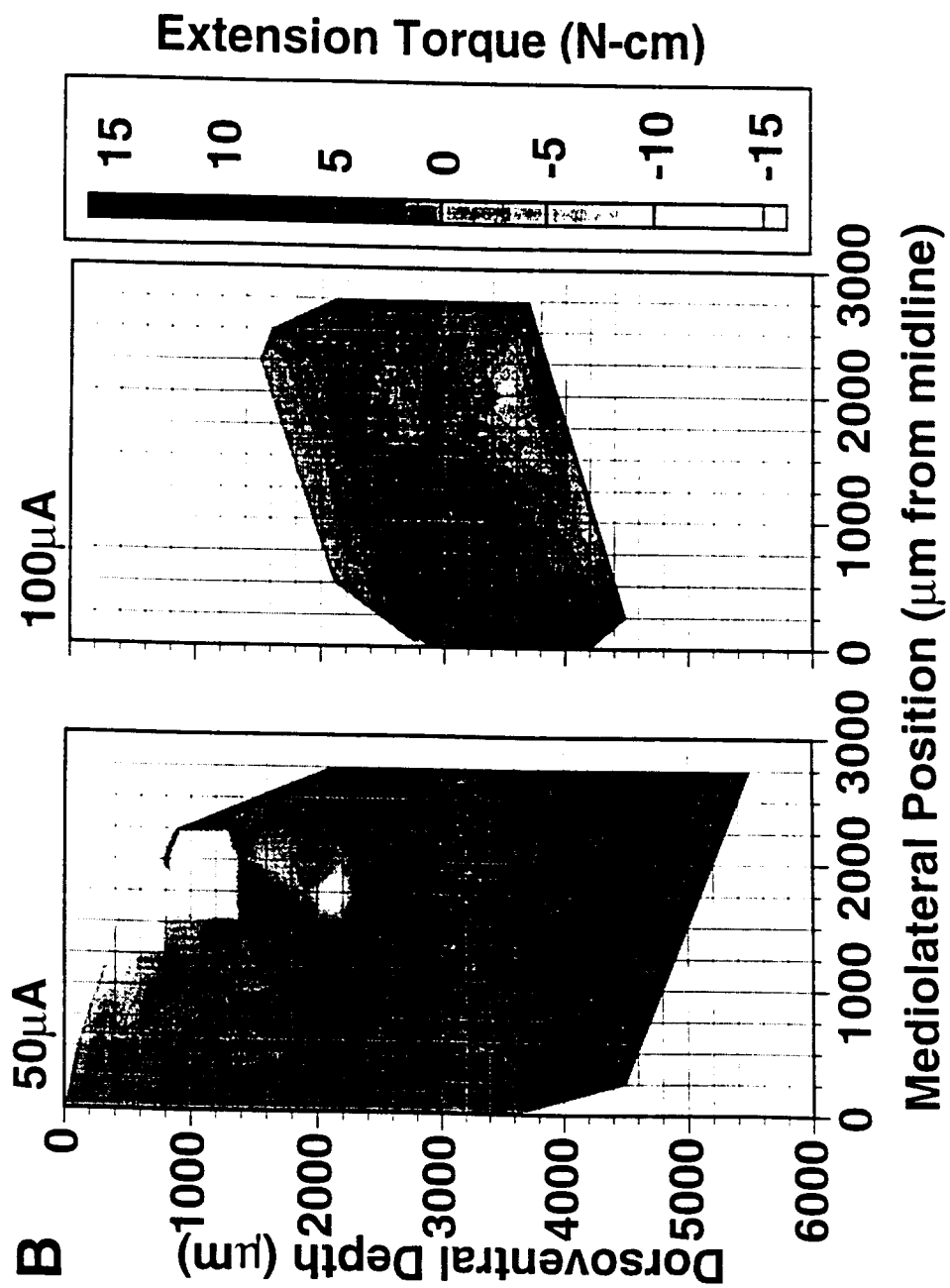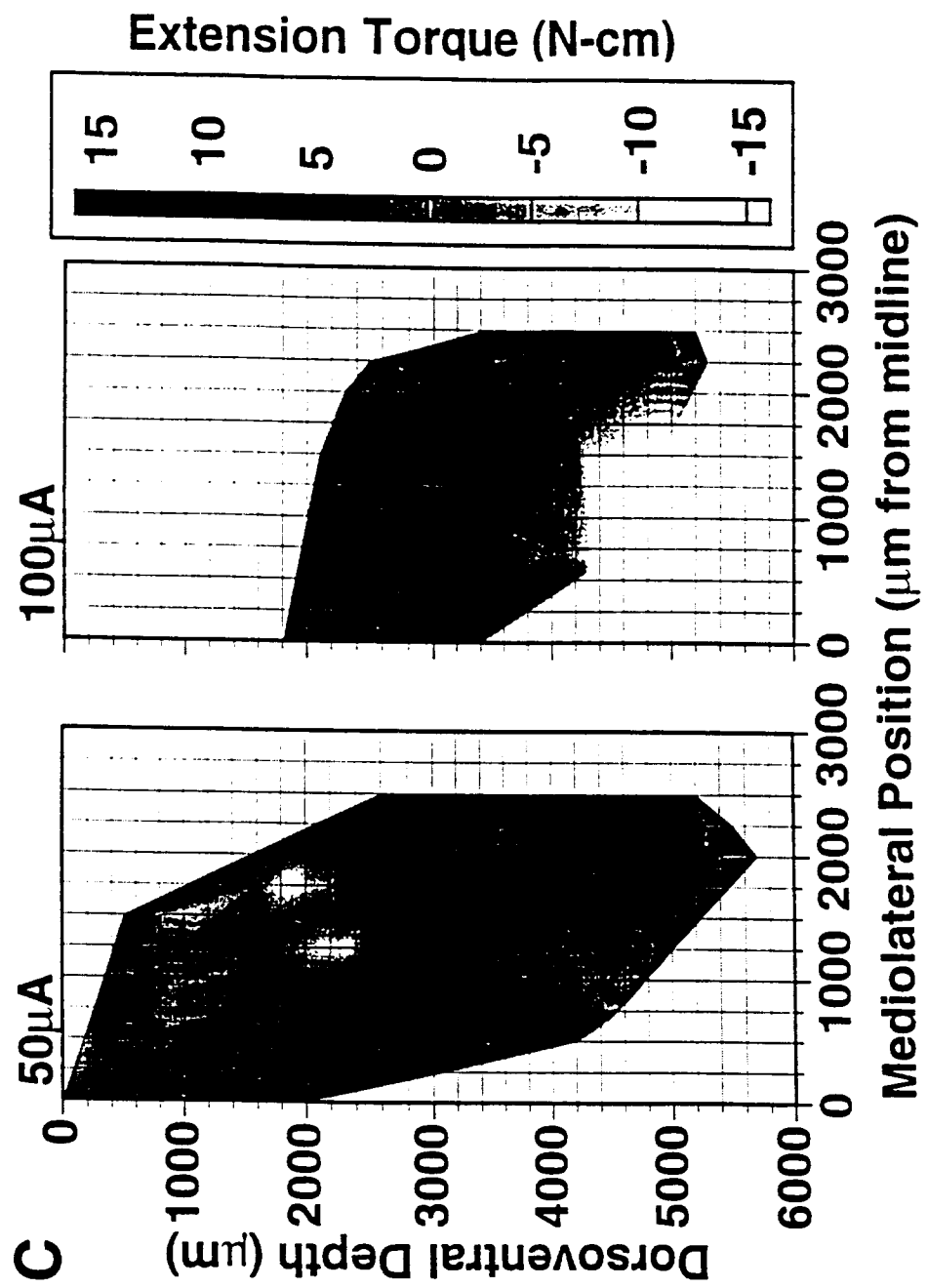
Figure 2: Photomicrograph of a hemisection of the S1 spinal cord from an animal undergoing 2 h of isometric micturition. Neurons exhibiting Fos-like immunoreactivity (FLI) appear as black dots, and are observed in the superficial dorsal horn, dorsal to the central canal, in a horizontal band extending from the central canal to the sacral parasympathetic nucleus (SPN), and within the SPN (intermediolateral cell column). Bar=500 µm.

Figure 3: ON FOLLOWING THREE PAGES
Two dimensional maps of the knee torques evoked by microstimulation of the lumbar spinal cord (1 s 20 Hz burst of 100 μs, 50 μA or 100 μA pulses). Each panel is a 2-dimensional map of the spinal cord at three different rostrocaudal levels: the middle of L5 (A), the L5/L6 border (B), and the middle of L6 (C). The evoked extension or flexion torques are plotted as grayscale values. Mediolateral zero corresponds to the midline, dorsoventral zero corresponds to the cord surface at the midline, and the intersections of the orthogonal grid indicate the points at which mapping stimuli were applied.
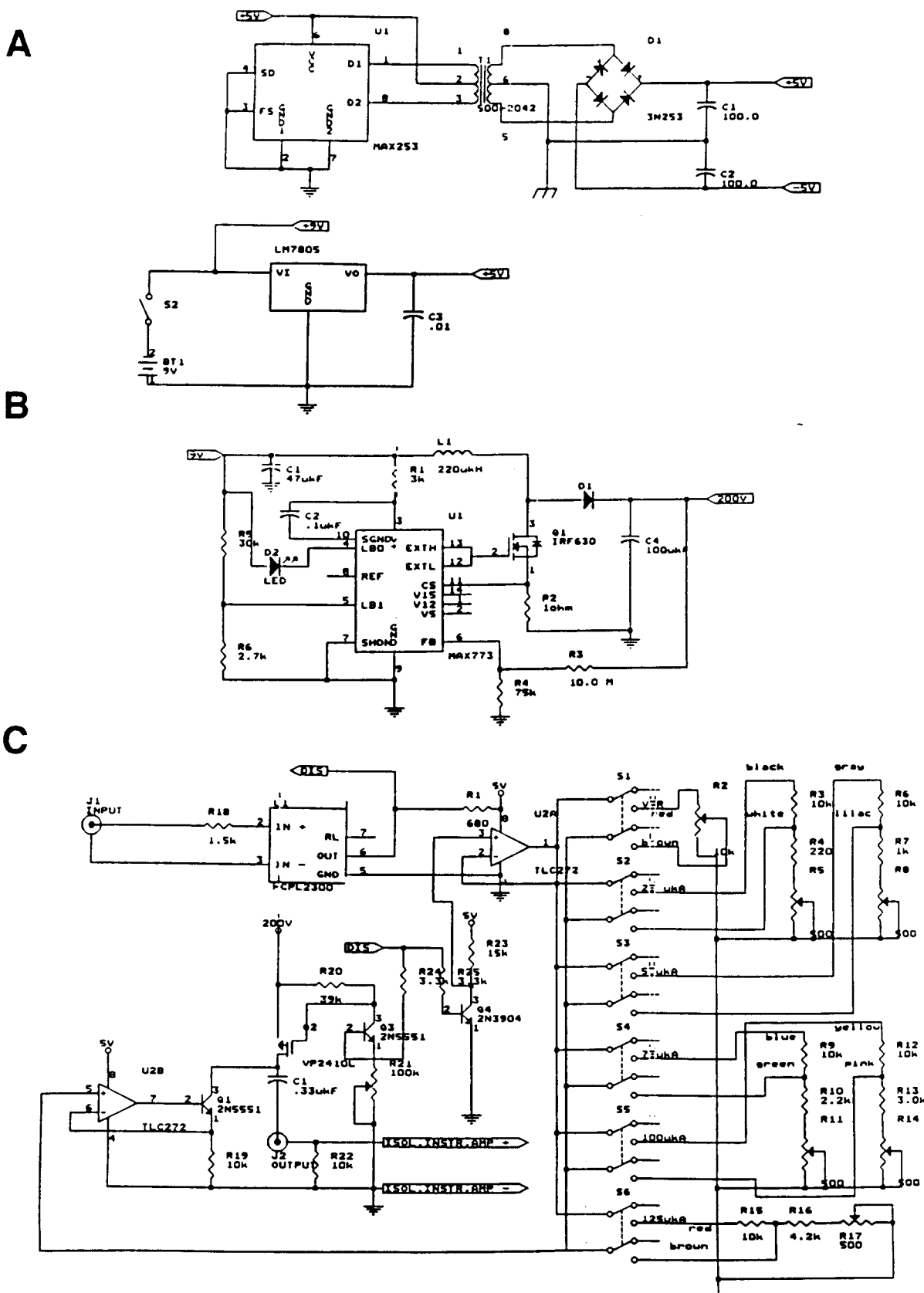
Extension Torque (N-cm)

15
10
5
0
-5
-10
-15

100μA

50μA

A

Dorsoventral Depth (μm)

0
1000
2000
3000
4000
5000

Mediolateral Position (μm from midline)

0
1000
2000
3000

Extension Torque (N-cm)

100μA

50μA

B

Dorsoventral Depth (μm)

Mediolateral Position (μm from midline)

Extension Torque (N-cm)

Mediolateral Position (μm from midline)

Dorsoventral Depth (μm)

50μA

100μA

C

Figure 4: Schematics for the circuit power supply (A), the output power supply (B), and the stimulator circuit (C) for the high compliance voltage constant current source.
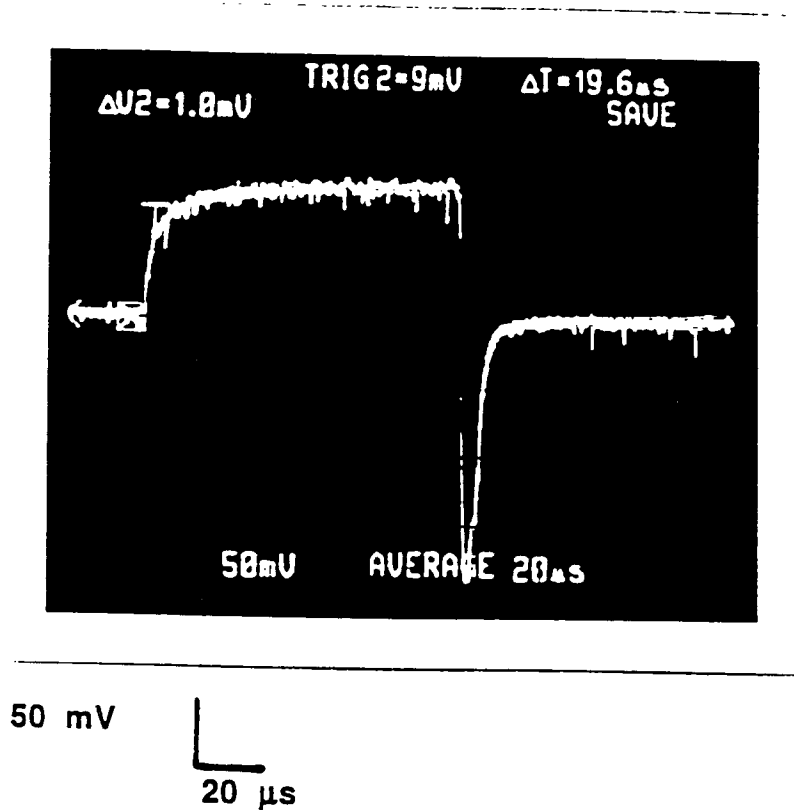
Figure 5: Photograph of an oscilloscope trace (50 mV/div vertical, 20 μs/div horizontal) of the output current (100 μA, 100 μs) into a 100 kΩ load, measured across a 1 kΩ resistor in series with the load.
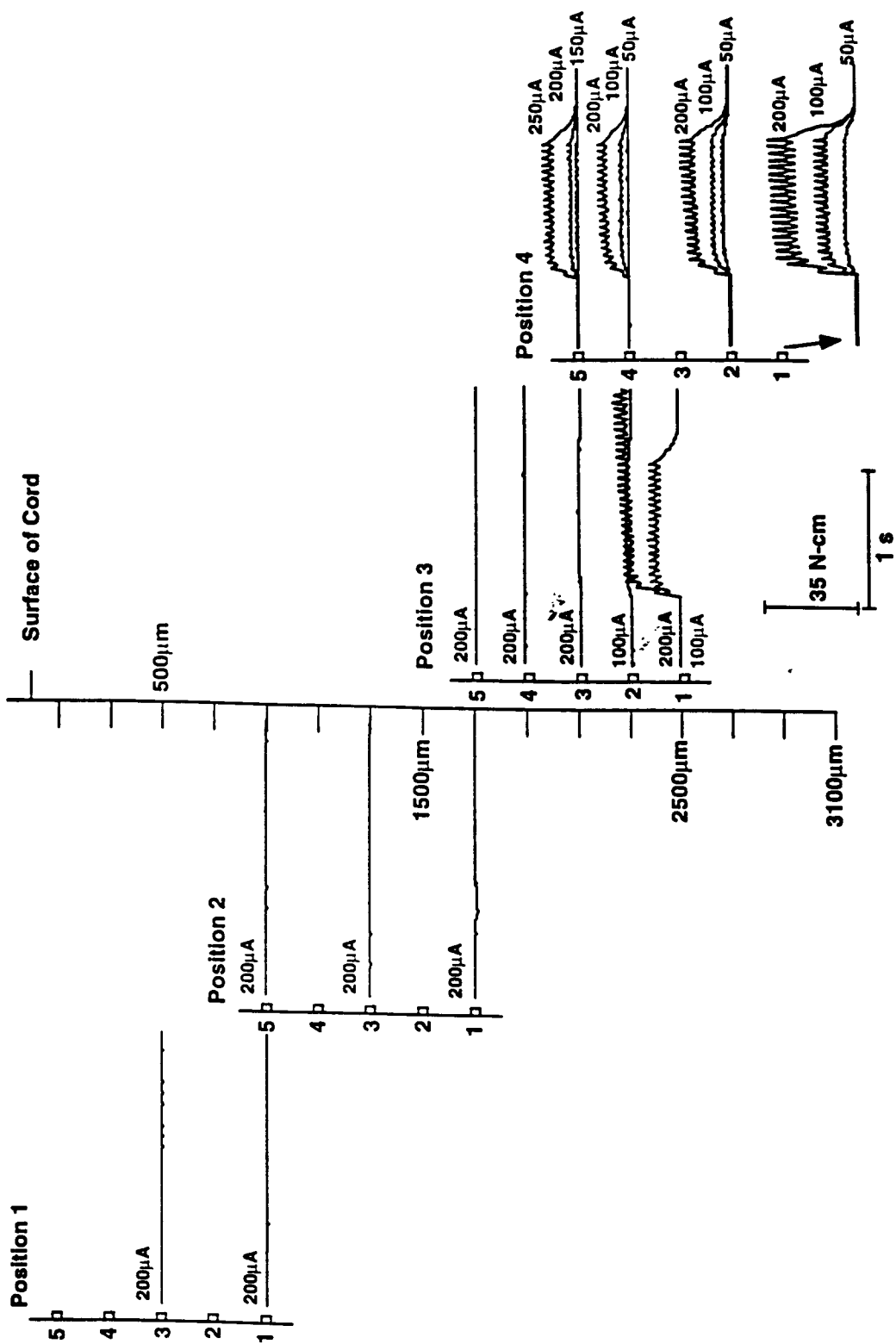
Figure 6